

The Product of the Vaccinia Virus L5R Gene Is a Fourth Membrane Protein Encoded by All Poxviruses That Is Required for Cell Entry and Cell-Cell Fusion

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The L5R gene of vaccinia virus is conserved among all sequenced members of the *Poxviridae* but has no predicted function or recognized nonpoxvirus homolog. Here we provide the initial characterization of the L5 protein. L5 is expressed following DNA replication with kinetics typical of a viral late protein, contains a single intramolecular disulfide bond formed by the virus-encoded cytoplasmic redox pathway, and is incorporated into intracellular mature virus particles, where it is exposed on the membrane surface. To determine whether L5 is essential for virus replication, we constructed a mutant that synthesizes L5 only in the presence of an inducer. The mutant exhibited a conditional-lethal phenotype, as cell-to-cell virus spread and formation of infectious progeny were dependent on the inducer. Nevertheless, all stages of replication occurred in the absence of inducer and intracellular and extracellular progeny virions appeared morphologically normal. Noninfectious virions lacking L5 could bind to cells, but the cores did not enter the cytoplasm. In addition, virions lacking L5 were unable to mediate low-pH-triggered cell-cell fusion from within or without. The phenotype of the L5R conditional lethal mutant is identical to that of recently described mutants in which expression of the A21, A28, and H2 genes is repressed. Thus, L5 is the fourth component of the poxvirus cell entry/fusion apparatus that is required for entry of both the intracellular and extracellular infectious forms of vaccinia virus.

Investigations of the mechanism(s) used by vaccinia virus, the prototype poxvirus, to enter cells are complicated by the existence of multiple infectious forms including intracellular mature virions, which are released by cell lysis; intracellular enveloped virions, which mediate intracellular transport; and extracellular virions, which are released from intact cells by exocytosis (35). Intracellular enveloped virions and extracellular virions are essentially intracellular mature virions with two or one additional outer membrane, respectively. There are two types of extracellular enveloped virions, cell-associated and released (3, 25). In most vaccinia virus strains, the former predominate and efficiently mediate cell-to-cell spread at the tips of actin-containing microvilli (39). The viral proteins in the outer membrane of intracellular mature virions and extracellular virions are entirely different and consequently bind differently to cells (42), although the receptors have not been identified.

Several mechanisms of vaccinia virus entry involving fusion of extracellular enveloped virion-specific membranes or intracellular mature virion membranes have been proposed (36). Furthermore, it has been suggested that the intracellular mature virion itself contains multiple membranes (15). The topological problems associated with the fusion of virions with multiple membranes have led to proposals of nonfusion mechanisms of entry (24). Because of space constraints, we are

unable to critically review the entire literature and consequently will summarize evidence that compels us to believe that the intracellular mature virion membrane consists of a single bilayer, which fuses with a cell membrane, and that the outer extracellular enveloped virion membrane is nonfusogenic. For contrary views, consult references 14, 15, 24, 28, and 37.

Numerous transmission electron micrographic images, prepared by independent laboratories (7, 16, 18), reveal a typical membrane bilayer delimiting immature and mature virions. Recently, the presence of a single outer membrane bilayer was confirmed by freeze fracture (17) and was consistent with cryo-electron tomography (6), although the latter study suggested an additional membrane around the core. The fusion of the intracellular mature virion membrane with the plasma membrane was demonstrated by electron microscopy (2, 4) and supported by evidence for incorporation of viral membrane proteins in the plasma membrane (22) and lipid mixing studies (10). In contrast, there is no evidence for fusion of the extracellular enveloped virion membrane, which is likely disrupted prior to or during virus entry. Three glycosaminoglycan-binding proteins (D8, H3, and A27) may facilitate initial binding of intracellular mature virions to the plasma membrane (5, 19, 23) but are not required for cell entry. Instead, three other intracellular mature virion membrane proteins (A28, H2, and A21) are not individually required for cell attachment but are needed for neutral pH entry and low-pH-induced cell-cell fusion mediated by intracellular mature virions as well as for cell-to-cell spread and fusion mediated by cell-associated extracellular enveloped virions (30, 32, 40). We suggested that

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the latter proteins form part of a fusion apparatus that is conserved in all members of the poxvirus family. Here, we provide evidence for an additional conserved intracellular mature virion membrane protein that is required for entry and fusion.

MATERIALS AND METHODS

Cells and viruses. All experiments were performed with the Western Reserve (WR) strain of vaccinia virus (ATCC VR-1354; accession number AY243312) or recombinant viruses derived from this strain. The amplification and titration of vaccinia virus WR and recombinant viruses was performed as previously described (11). For the propagation of vV5-L5i, HeLa S3 cells (ATCC CCL-2.2) were incubated in the presence of 50 μ M isopropyl- β -D-thiogalactopyranoside (IPTG) for 48 h at 37°C. For purification of intracellular mature virions, virus was amplified in HeLa S3 cells in the presence or absence of IPTG and purified by sedimentation through two sucrose cushions and one sucrose gradient as previously described (11). The purity of the preparations was determined by electron microscopy and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The particle/PFU ratio was determined by optical density at 260 nm and plaque titration.

Recombinant virus construction. vV5-L5i was derived from vT7LacOI, a recombinant vaccinia virus possessing an IPTG-inducible T7 RNA polymerase gene in addition to an *Escherichia coli lac* repressor gene situated within the dispensable thymidine kinase locus (1). The protocol for vV5-L5i construction was similar to that used to produce vE10Li (33). Briefly, a PCR product was generated that encompassed the entire vaccinia virus L5R open reading frame (ORF) (nucleotides 79904 to 80290) under the control of a *lac* operator-regulated T7 promoter containing a consensus sequence for initiation of translation (CGAAATTAATACGACTCACTATAGGGAATTGTGAGCGCTCACAATTCCCGCCGCCACCATG), adjacent to a copy of the enhanced green fluorescent protein gene (EGFP) (accession no. AAG27429) under the control of a vaccinia virus synthetic early-late promoter (AAAAATTGAAATTTATTTTTTTTTTTTGGAATATAAATG). Nucleotides corresponding to the site of translation initiation for the respective ORFs are underlined. To ensure efficient homologous recombination, flanking sequences of approximately 650 bp were appended to both termini of the PCR product by recombinant PCR (Accuprime Pfx, Invitrogen). The final PCR product used for transfection was gel-purified (QIAquick gel extraction kit, QIAGEN) and verified by sequencing. BS-C-1 cells in 24-well plates were infected with 1 PFU per cell of vT7LacOI in Optimem (Invitrogen) 2 h prior to transfection with 0.3 μ g of purified PCR product (Lipofectamine 2000; Invitrogen). Transfected cells were harvested 24 h postinfection and subjected to three freeze-thaw cycles in a dry-ice bath. Tenfold dilutions of the infected-cell lysates were reseeded onto fresh BS-C-1 monolayers and incubated in the presence of 50 μ M IPTG. Isolated viral plaques exhibiting enhanced green fluorescent protein (EGFP) expression were clonally purified by a further five rounds of single plaque selection in the presence of 50 μ M IPTG. After virus propagation, we noted the presence of a low level (~0.5%) of "revertant" virus, which expressed GFP but made large plaques in the absence of IPTG.

vV5-L5 was constructed from vV5-L5i, using a protocol similar to that described above. The recombinant PCR product used for transfection (nucleotides 79280 to 80572) comprised a region of the vT7LacOI genome encompassing the entire L5R ORF, the putative L5R promoter, flanking sequences to facilitate homologous recombination, and codons corresponding to a V5 epitope tag sequence (amino acid sequence: GKIPNPLLGLDST) fused to the region of the 5' terminus of the L5R ORF. Following transfection of vV5-L5i-infected cells, the cells were harvested and subjected to three freeze-thaw cycles and sonication. Tenfold dilutions of the infected-cell lysate were reseeded onto fresh BS-C-1 monolayers without IPTG in the medium to prevent replication of the parental virus. Isolates exhibiting a large-plaque phenotype without EGFP expression were clonally purified by a further five rounds of single plaque selection in the absence of IPTG. All genomic rearrangements were confirmed by PCR and sequencing of viral DNA.

Antibodies. The following mouse monoclonal antibodies were used: 7D11 to the L1 protein (43) and anti-V5 (Invitrogen). Rabbit polyclonal antibodies recognizing the following vaccinia virus proteins were used: L1 (27) and A4 (9). For detection of cell-associated extracellular enveloped virions, rat anti-B5 monoclonal antibody (19C2) was used (29). Cy5-conjugated donkey anti-rat antibody, fluorescein isothiocyanate-conjugated goat anti-mouse antibody, and rhodamine red-X-conjugated goat anti-rabbit antibody were purchased from Jackson ImmunoResearch and used according to the manufacturer's directions.

Western and Northern blotting. Unless otherwise noted, the procedures were similar to those described (40).

Trypsin treatment of purified virions. Purified virions were incubated in the presence of 50 mM Tris, 10 mM CaCl₂, pH 8, and incubated for 30 min at 37°C. Porcine trypsin (Sigma) was added to a final concentration of 250 μ g/ml and incubated for 30 min at 37°C. Digestions were terminated by addition of soybean trypsin inhibitor (Sigma) to a final concentration of 500 μ g/ml. The virions were centrifuged at 20,000 $\times g$ for 30 min at 4°C, and the pellet and supernatant fractions were resuspended in lithium dodecyl sulfate sample loading buffer (Invitrogen) and incubated at 95°C for 5 min prior to SDS-PAGE and Western blotting. As a positive control for trypsin digestion, purified virions were resuspended in buffer (50 mM Tris, 10 mM CaCl₂, [pH 8]) containing 1% NP-40 and incubated for 30 min at 37°C. Following this incubation, trypsin was added to a final concentration of 250 μ g/ml and the incubation was continued for 30 min at 37°C. Trypsinization reactions were terminated as described above, and the pellet and supernatant fractions were examined by Western blotting.

Transient expression of L5. A PCR product consisting of the entire L5R gene under the control of its native promoter with the addition of codons specifying a C-terminal V5 tag was generated with the following primer pair: GTACTAAT AAGGAAGTGAATCGTATAGTTCTAG (nucleotides 79764 to 79797) and GGGGTCACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCTCTGCGAAGAATCATCGTTAAGAGATACTGG (nucleotides 80301 to 80329). Nucleotides noncomplementary to the vaccinia virus WR sequence are underlined. The PCR product was amplified with Accuprime Pfx polymerase using purified vaccinia virus DNA as a template and purified with a QIAGEN PCR purification kit and subcloned into pCR-Blunt II-TOPO (Invitrogen). For transfection, BS-C-1 cells were infected with vE10i or vT7LacOI at a multiplicity of 5 PFU per cell. After 2 h, the cells were transfected with 0.25 μ g of vector DNA (Lipofectamine 2000, Invitrogen). At 18 h postinfection, cells were pelleted, washed in sterile phosphate-buffered saline, and incubated with alkylating agents.

RESULTS

Expression of the L5R gene product. The L5R gene VACWR092 of vaccinia virus is conserved among all sequenced members of the *Poxviridae*, suggesting that it has an important role in virus replication. A multiple sequence alignment, including an ortholog from each known genus of vertebrate and invertebrate poxviruses, is presented in Fig. 1. L5R orthologs encode two conserved cysteine residues and an N-terminal stretch of hydrophobic amino acids predicted to function as a transmembrane anchor. We thought that the L5 protein (note that we distinguish between vaccinia virus proteins from genes and ORFs by dropping the L or R, which signifies left or right direction of transcription) would be expressed after DNA replication, as the sequence upstream of the L5R ORF contains the late promoter consensus motif (8).

To determine the kinetics of expression, we constructed a recombinant virus (vV5-L5) in which the coding sequence for the 15-amino-acid V5 epitope tag was fused to the start of the L5R ORF, which was maintained under the control of its native vaccinia virus promoter. Inclusion of the V5 epitope tag did not affect viral replication, as plaque size and virus yield during single-step replication were comparable to that of an unmodified virus (data not shown). Lysates of vV5-L5-infected BS-C-1 cells contained a 15-kDa protein that reacted by Western blotting with a monoclonal antibody to the V5 epitope tag (Fig. 2A), consistent with the predicted mass of L5. The 15-kDa protein was detected at 6 h postinfection and increased in amount until 24 h. At that time, there was a minor 32-kDa species, the significance of which is not known. When 1- β -D-arabinofuranosylcytosine (AraC) was used to inhibit viral DNA replication, the 15-kDa protein was not detected. Thus, the kinetics of expression and the requirement for viral DNA

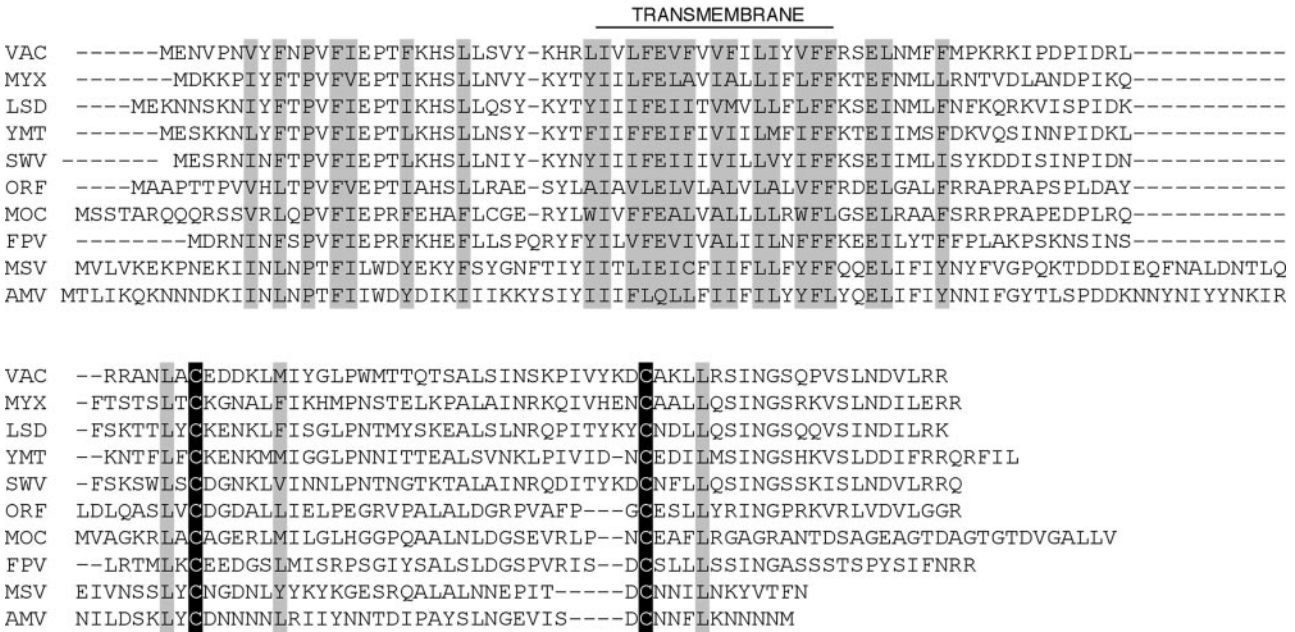


FIG. 1. L5 is conserved among members of the *Poxviridae* family. (A) Multiple sequence alignment of L5 orthologs, comprising a representative sequence from each genus of the *Chordopoxvirinae* and two available sequences from *Entomopoxvirinae*. Invariant cysteines are identified by white text on a black background; other invariant or similar residues in all aligned sequences are identified by shading. Abbreviations: VAC, vaccinia virus (*Orthopoxvirus*); MYX, myxomavirus (*Leporipoxvirus*); LSD, lumpy skin disease virus (*Capripoxvirus*); YMT, Yaba monkey tumor virus (*Yatapoxvirus*); SWV, swinepox virus (*Suipoxvirus*); ORF, ORF virus (*Parapoxvirus*); MOC, molluscum contagiosum virus (*Molluscipoxvirus*); FWP, fowlpox virus (*Avipoxvirus*); AMV, *Amsacta moorei* entomopoxvirus (*Entomopoxvirus B*); MSV, *Melanoplus sanguinipes* entomopoxvirus (*Entomopoxvirus B*).

synthesis were consistent with regulation of the L5R gene by a late promoter.

L5 contains one intramolecular disulfide bond. All L5R orthologs encode two conserved cysteine residues that could potentially form inter- or intramolecular disulfide bonds. In-

termolecular disulfides, however, were ruled out as the gel electrophoretic mobility was appropriate for a 15-kDa protein and there was no appreciable difference in mobility between unreduced and reduced L5 (Fig. 2B). To ascertain whether L5 contains intramolecular disulfides, BS-C-1 cells infected with

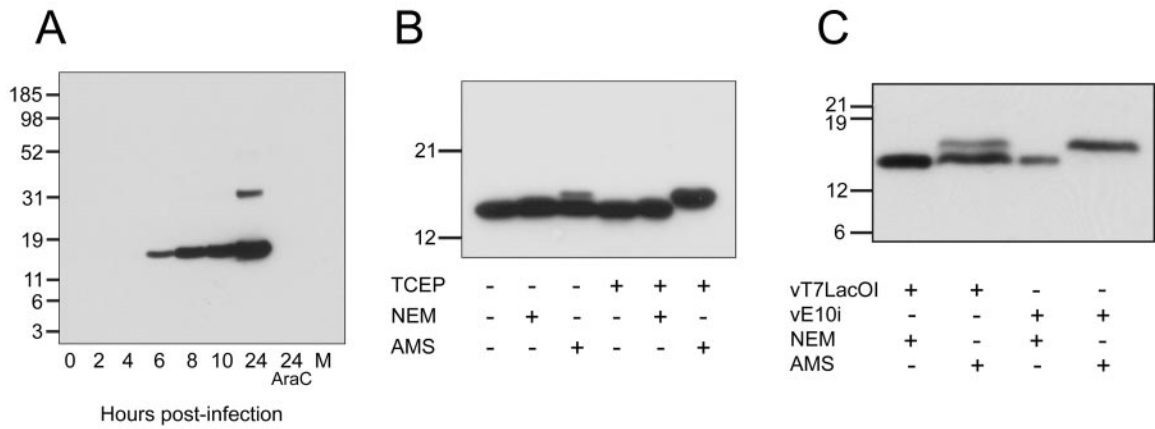


FIG. 2. L5 is synthesized with the kinetics of a typical late protein and contains an intramolecular disulfide bond formed by the vaccinia virus-encoded redox pathway. (A) Kinetics of L5 protein accumulation. BS-C-1 cells were mock infected (M) or infected with 5 PFU of vV5-L5 in the absence or presence of AraC and lysates were analyzed by SDS-PAGE and Western blotting at the indicated times postinfection. Cell lysates were subject to SDS-PAGE and Western blot analysis using an anti-V5 monoclonal antibody. (B) Evidence for an intramolecular disulfide bond. BS-C-1 cells were infected with 5 PFU per cell of vV5-L5 and incubated for 18 h. Cells were disrupted in SDS-PAGE loading buffer containing *N*-ethylmaleimide (NEM) or AMS. Alternatively, cells were treated with the reducing agent Tris-(2-carboxyethyl)phosphine (TCEP) prior to alkylation with either *N*-ethylmaleimide or AMS. Cell lysates were subjected to SDS-PAGE and Western blotting using an anti-V5 monoclonal antibody. (C) Disulfide bond formation requires the vaccinia virus E10 protein. BS-C-1 cells were infected for 2 h with either vE10i or vT7LacOI as a control. The cells were then transfected with a plasmid containing the L5R ORF with a C-terminal V5 epitope tag under the control of the natural L5R promoter. At 18 h postinfection, cells were disrupted in SDS-PAGE loading buffer containing *N*-ethylmaleimide or AMS. Cell lysates were subjected to SDS-PAGE and Western blot analysis using an anti-V5 monoclonal antibody. Mass standards, shown on the vertical axis, are in kDa.

vV5-L5 were lysed in the presence of the alkylating agent *N*-ethylmaleimide or 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS), which increase protein mass by 0.125 or 0.536 kDa per reactive cysteine, respectively. Upon treatment with AMS, only a minor amount of L5 shifted in mobility, indicating that most of the protein lacked reactive cysteines. This result should be compared with the effect of first reducing the protein with Tris-(2-carboxyethyl)phosphine prior to AMS treatment: a complete ~1-kDa shift in mobility occurred (Fig. 2B). Thus, the majority of L5 contains an intramolecular disulfide bond.

Vaccinia virus-encoded redox pathway is required for the formation of the L5 disulfide bond. The vaccinia virus genome encodes three oxidoreductases (E10, A2.5, and G4) that act in series to promote the formation of intramolecular disulfides within the cytoplasmic domains of several intracellular mature virion membrane proteins (34). The absence of any one of these redox proteins results in a failure to catalyze intramolecular disulfide formation. The following scheme was devised to determine whether L5 disulfide bond formation requires the vaccinia virus-encoded redox pathway. We transfected a plasmid encoding L5 with a C-terminal V5 epitope tag, under the control of its native promoter, into BS-C-1 cells infected with the conditional-lethal vaccinia virus mutant vE10i. In vE10i, the E10R gene is regulated by an *E. coli lac* operator and is not expressed in the absence of an inducer. Cells infected with VT7lacOI, the parent of vE10i with an unmodified E10 gene, were transfected in parallel. The alkylation protocol described in the previous section was used to distinguish disulfide-bonded and free sulfhydryl groups. In cells infected with vE10i in the absence of inducer, L5 was completely reduced, as demonstrated by the complete mobility shift upon alkylation with AMS (Fig. 2C). In cells infected with the vT7LacOI control virus and transfected with the L5 plasmid under the same conditions, however, only a minority of L5 was reduced (Fig. 2C). Overexpression may have contributed to the amount of reduced L5 in vT7LacOI-infected cells, as this has been noted when other vaccinia virus genes were expressed by transfection (31). These experiments demonstrate that the vaccinia virus-encoded redox pathway is required for the formation of the disulfide bond in L5.

L5 is associated with the intracellular mature virion membrane. The association of L5 with sucrose gradient-purified intracellular mature virions was demonstrated by Western blotting using antibody to the V5 epitope tag. Some L5 was released with the nonionic detergent NP-40, but dithiothreitol was also required to release most of the protein (Fig. 3A). The conditions required for release of L5 closely resembled those for L1, a well-characterized intracellular mature virion membrane protein (Fig. 3A).

We predicted that L5 is anchored in the viral membrane by the N-terminal hydrophobic domain (Fig. 1) with the large C-terminal fragment exposed on the surface of the intracellular mature virions and the smaller N-terminal epitope-tagged segment beneath the membrane. Sensitivity to trypsin and reactivity with a membrane-nonpermeating biotinylation reagent were the two methods used to investigate the membrane topology of L5. Purified vV5-L5 intracellular mature virions were incubated with trypsin, and the pellet and supernatant fractions were analyzed by Western blotting. Anti-V5 antibody

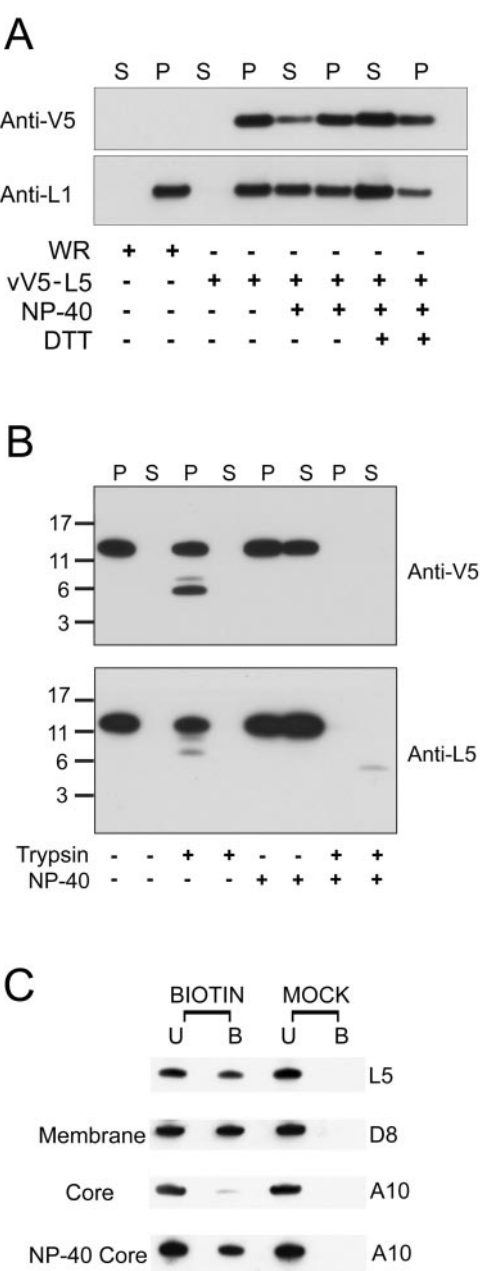


FIG. 3. L5 is an integral membrane protein exposed on the surface of intracellular mature virions. (A) Purified vaccinia virus WR or vV5-L5 intracellular mature virions were suspended in Tris buffer, pH 7.4, and incubated at 37°C for 30 min, with or without the addition of 1% NP-40 or 50 mM dithiothreitol (DTT) as indicated. After 30 min, virus suspensions were centrifuged at 20,000 × g for 30 min at 4°C and separated into pellet (P) and supernatant (S) fractions. The separate fractions were solubilized in SDS-PAGE loading buffer and the resulting lysates were subject to SDS-PAGE and Western blotting using anti-V5 and anti-L1 antibodies. (B) Purified vV5-L5 intracellular mature virions were treated with trypsin, NP-40, or NP-40 plus trypsin. Pellet and supernatant fractions were analyzed by SDS-PAGE. Mass markers, shown on the vertical axis, are in kDa. (C) Biotinylation of surface proteins. Purified intact or NP-40-disrupted vV5-L5 intracellular mature virions were treated or mock treated with sulfo-NHS-SS-biotin as previously described (40). After SDS dissociation, the proteins were incubated with neutravidin beads and bound (B) and unbound (U) proteins were analyzed by SDS-PAGE followed by Western blotting with antibodies to the V5 epitope tag to reveal L5 or to the D8 surface membrane protein or the A10 core protein. The analysis of biotinylated D8 and A10 was presented previously (40).

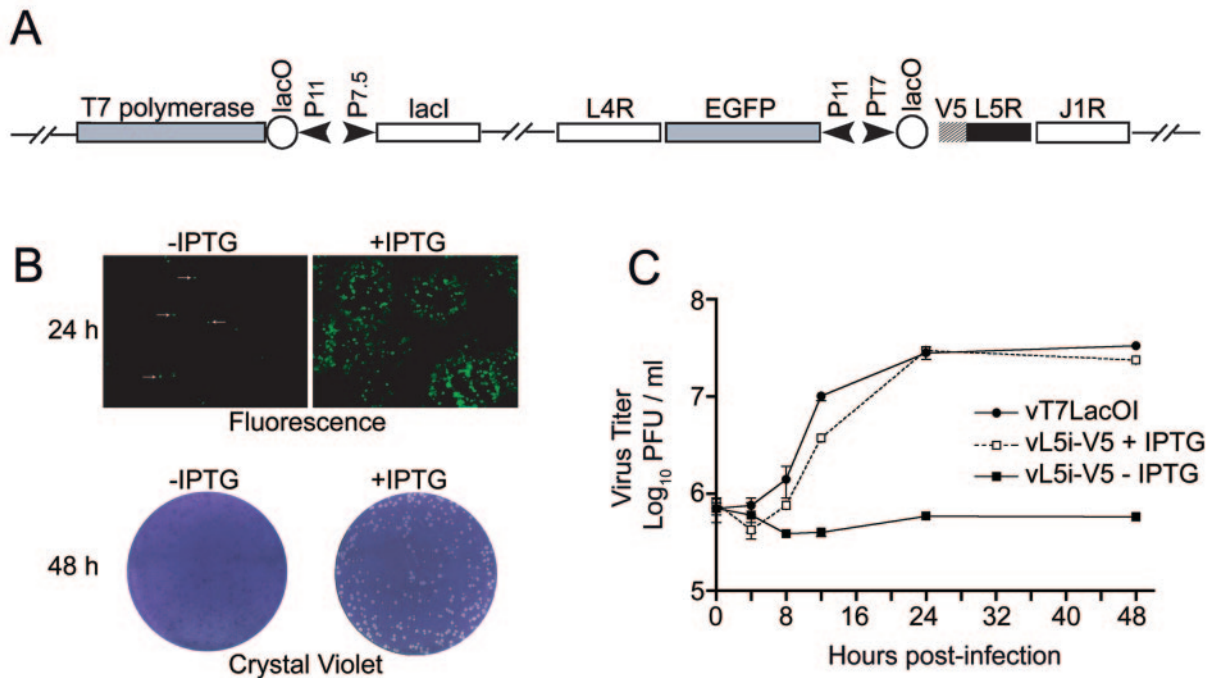


FIG. 4. Conditional lethal phenotype of a recombinant vaccinia virus with an inducible L5R gene. (A) Schematic of portions of the vV5-L5i genome. Abbreviations: *lacO*, *E. coli lac* operator; P₁₁, vaccinia virus late promoter; P_{7.5}, vaccinia virus early/late promoter; *lacI*, *E. coli lac* repressor gene; P_{T7}, bacteriophage T7 promoter. (B) Plaque formation. BS-C-1 monolayers were infected with vV5-L5i in the presence (+) or absence (-) of IPTG. At 24 h postinfection, monolayers were examined by fluorescence microscopy for the presence of EGFP. Arrows point to fluorescent cells. At 48 h, monolayers were fixed and stained with crystal violet. (C) Single-step virus yields. BS-C-1 monolayers were infected with 5 PFU per cell of the indicated virus in the presence (+) or absence (-) of IPTG. The cells were harvested at the indicated hours postinfection, and virus titers were determined by plaque assay in the presence of 50 μ M IPTG. The experiment was performed in duplicate, and the data points represent the mean \pm standard error of the mean. At some points, the error bars are too close to resolve.

recognized a \sim 6-kDa cleavage product in addition to un-cleaved L5 in the pellet fraction of trypsin-treated vV5-L5 virions (Fig. 3B). The estimated mass of \sim 6 kDa was close to the calculated 7.3-kDa mass of the V5-tagged peptide that would remain associated with the viral membrane after cleavage at the first arginine predicted to be external to the trans-membrane domain. The failure of the 6-kDa product to react with an antibody to the L5 peptide MPKRKIPDPIDRLR (Fig. 3B), which starts 8 amino acids downstream of the arginine, was consistent with our interpretation. Cleavage at the next trypsin-sensitive site would result in a product \sim 1 kDa larger, perhaps accounting for the minor band reactive with anti-V5 and anti-L5 antibodies. Less than half of the membrane-associated L5 was digested with trypsin, whereas complete digestion occurred after NP-40 treatment, suggesting that the cleavage site was incompletely exposed. Under the same conditions of trypsin treatment, the A27 surface protein was completely digested (data not shown).

Further evidence for the presence of L5 on the intracellular mature virion surface was obtained by biotinylation with sulfo-succinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin), a membrane-nonpermeating reagent that selectively reacts with primary amines. Purified virions were treated or mock treated with sulfo-NHS-SS-biotin. After blocking excess reagent, the proteins were dissociated with SDS and incubated with neutravidin beads, which bind biotin. The bound and unbound proteins were analyzed by Western blotting. Binding was

specific, as the proteins from mock-treated virions were entirely in the unbound fraction (Fig. 3C). In contrast, L5 was biotinylated and the proportion was similar to that of the D8 surface membrane protein and considerably more than A10, a core protein (Fig. 3C). An additional control showed that A10 was biotinylated after NP-40 extraction of the membrane.

L5 is required for plaque formation and infectious virion production. To determine whether L5 is essential for replication, we constructed a mutant virus, called vV5-L5i, which requires IPTG for expression of L5. As shown in Fig. 4A, the V5 epitope-tagged L5R ORF is regulated by a bacteriophage T7 promoter and an *Escherichia coli lac* operator. In addition, the recombinant virus contains the T7 DNA-dependent RNA polymerase gene regulated by a vaccinia virus late promoter and a *lac* operator as well as a constitutively expressed *E. coli lac* repressor. In the absence of IPTG, the *lac* repressor should bind to both operators and stringently prevent expression of L5. However, when IPTG is added, the repression should be relieved and L5 expressed.

In the absence of IPTG, vV5-L5i plaque formation was reduced 99.5% compared to 50 μ M IPTG (Fig. 4B), which was shown to be optimal. Upon analysis by fluorescence microscopy, isolated EGFP-positive cells were detected in the absence of IPTG, demonstrating that vV5-L5i had a defect resulting in failure of cell-to-cell spread. (To understand this and succeeding experiments, it is important to recall that vV5-L5i stocks are prepared in the presence of IPTG and that conse-

quently the infecting virus contains L5 even though little or no additional L5 will be made in the absence of IPTG.) Moreover, infectious virus production was prevented in the absence of IPTG, as shown by a one-step growth analysis (Fig. 4C). The yield of infectious virus produced in the presence of IPTG was similar to that of the parental virus, vT7lacOI, which contains an unmodified L5R gene.

L5 is not required for assembly of virus particles. Repression of many intracellular mature virion membrane proteins results in a block in virus assembly. To determine whether L5 is required for formation of virions, cells were infected with vV5-L5i in the presence or absence of IPTG and cell sections were analyzed by transmission electron microscopy. Viral morphogenesis appeared entirely normal in the presence or absence of IPTG, resulting in the formation of mature intracellular and extracellular virions that were indistinguishable from wild-type virions (not shown). Moreover, extracellular virions that formed in the presence or absence of IPTG were associated with actin tails (Fig. 5), which are required for efficient cell-to-cell spread. A similar phenotype had previously been found when expression of A28, H2, and A21 was repressed.

Morphology, protein composition, and infectivity of virions lacking L5. The vV5-L5i stocks used in all experiments up to this point were generated in the presence of IPTG and, as has been noted, contained L5. To compare intracellular mature virions lacking L5 (–L5) and containing L5 (+L5), we purified virus from HeLa cells that were infected with vV5-L5i in the absence or presence of IPTG, respectively. The +L5 and –L5 virions were indistinguishable by electron microscopy (Fig. 6A) and SDS-PAGE analysis (Fig. 6B). Nevertheless, there was more than a 40-fold difference in their specific infectivity as determined by optical density and plaque titration in the presence of IPTG, sufficient to explain the reduced yield of infectious virus in single-step growth experiments (Fig. 4C). The slight infectivity of purified –L5 virus could be due to residual inoculum +L5 virus that could not be completely removed by washing the cells after the adsorption period and a low (~0.5%) level of “revertant” virus, which made large plaques in the absence of IPTG.

Because L5 could not be detected in +L5 virions by silver-staining gels, Western blot analysis was used to confirm that L5 was present in +L5 virions but absent in –L5 virions (Fig. 6C). In addition, –L5 and +L5 virions contained similar levels of A28 and A21 (Fig. 6C), demonstrating that L5 is not required for other members of the putative poxvirus cell entry/fusion apparatus to associate with intracellular mature virions.

–L5 virions are unable to mediate core release into the cytosol. The localization of L5 to the intracellular mature virion membrane suggests a role for L5 during early events in vaccinia virus infection, such as cell attachment or entry. As viral cores are deposited in the cytoplasm following fusion of the intracellular mature virion membrane with the cell, we initially tested for the presence of cytoplasmic cores by measuring viral RNA synthesis. Viral RNA synthesis is initiated shortly after cell penetration, since all the necessary components for transcription are packaged within the virion core. Cells were infected in the presence of AraC, and viral RNA synthesis was analyzed by Northern blotting with labeled DNA probes complementary to the viral growth factor (C11R) or DNA polymerase (E9L) gene. C11R and E9L were transcribed abundantly in +L5-infected cells, however, the C11R and E9L transcripts were barely detectable in –L5-infected cells (Fig. 7A). Actin mRNA served as a control for RNA loading and integrity. To exclude the possibility that –L5 virions cannot synthesize viral RNA because the cores are inactive, we measured *in vitro* RNA synthesis by NP-40-permeabilized virions. We found that the transcriptional activities of purified +L5 and –L5 virions were comparable and proportional to the virion concentration (Fig. 7B).

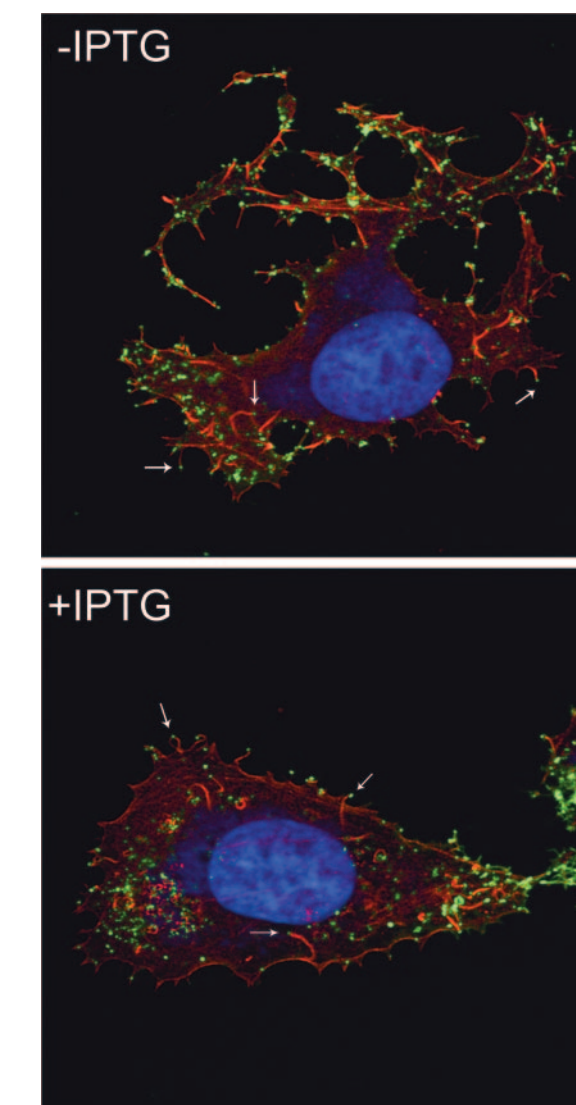


FIG. 5. vV5-L5i produces enveloped virions with actin tails in the absence of IPTG. HeLa cell monolayers were infected with 5 PFU per cell of vV5-L5i and incubated in the absence (–) or presence (+) of IPTG for 20 h. The infected cells were fixed with 3% paraformaldehyde and quenched with 2% glycine. Cell surface cell-associated extracellular enveloped virions were labeled with anti-B5R monoclonal antibody and Cy5-conjugated goat anti-rat secondary antibody. Following this, cells were permeabilized by the addition of 0.1% Triton X-100 and stained with DAPI to visualize nuclei and Alexa Fluor 568-phalloidin to visualize filamentous actin. Arrows point to representative cell-associated extracellular enveloped virions at the tips of actin tails.

dantly in +L5-infected cells, however, the C11R and E9L transcripts were barely detectable in –L5-infected cells (Fig. 7A). Actin mRNA served as a control for RNA loading and integrity. To exclude the possibility that –L5 virions cannot synthesize viral RNA because the cores are inactive, we measured *in vitro* RNA synthesis by NP-40-permeabilized virions. We found that the transcriptional activities of purified +L5 and –L5 virions were comparable and proportional to the virion concentration (Fig. 7B).

The above data suggested that the reduction in viral RNA

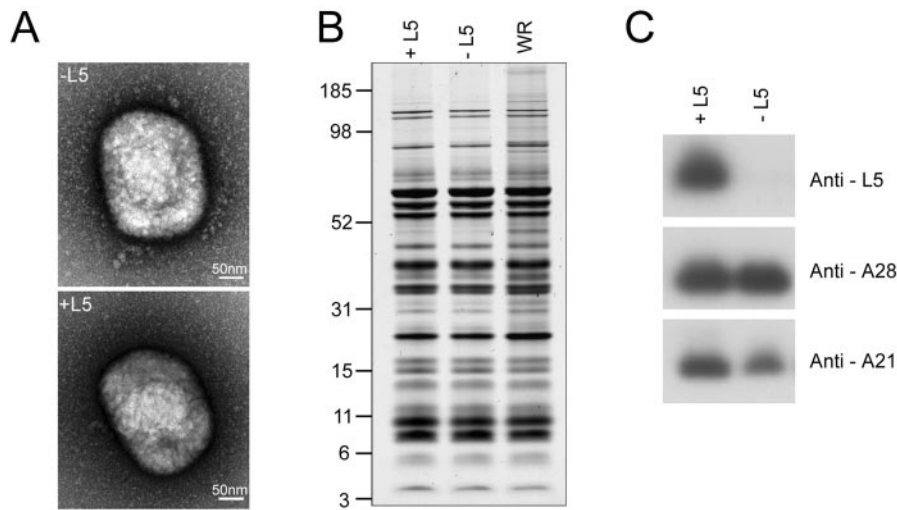


FIG. 6. Morphology and polypeptide composition of virions lacking or containing L5. (A) Electron microscopy of purified virions. Intracellular mature virions were purified by sucrose gradient sedimentation from cells infected with vV5-L5i in the presence (+ L5) or absence (–L5) of IPTG. Virions were deposited on grids, washed with water, and stained with 7% uranyl acetate in 50% ethanol for 30 seconds. (B) SDS-PAGE. Equivalent amounts of sucrose gradient-purified –L5, +L5, and vaccinia virus WR virions were solubilized in SDS-PAGE loading buffer and subjected to SDS-PAGE and silver staining. Mass markers are indicated in kDa on the left. (C) Western blotting. SDS-PAGE was performed as above, and the proteins were transferred to a membrane and probed with antibodies to the A28, A21, and L5 proteins.

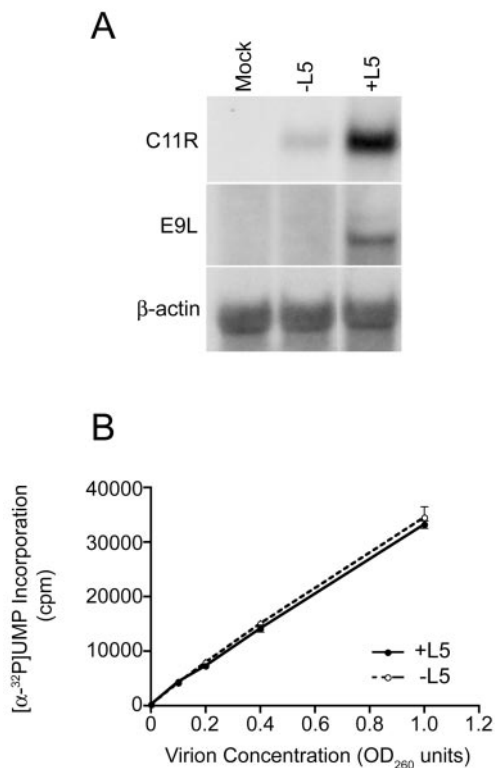


FIG. 7. Cells infected with –L5 virions exhibit reduced early-gene expression. (A) Northern blot analysis. BS-C-1 cell monolayers were mock infected or infected with 5 PFU per cell of +L5 virions or the corresponding OD_{260} of –L5 virions. Total RNA was harvested 3 h postinfection and subjected to Northern blot analysis with [α - 32 P]dCTP-labeled double-stranded DNA probes specific for the vaccinia virus growth factor (C11R), DNA polymerase (E9L), or cellular actin transcripts. (B) In vitro RNA synthesis. Purified +L5 and –L5 virions were permeabilized with NP-40 and incubated with ribonucleoside triphosphates and [α - 32 P]UTP. The incorporation of [α - 32 P]UMP into trichloroacetic acid-insoluble material was determined by scintillation counting and is expressed as counts per minute (cpm).

synthesis resulted from a failure of –L5 virions to penetrate cells. To evaluate this directly, we adapted the confocal microscopic assay of Vanderplassen et al. (42), which uses antibodies to envelope and core proteins to discriminate between detergent-permeabilized intracellular mature virions on the cell surface and cytoplasmic cores. In our adaptation, we used a monoclonal antibody to the L1 membrane protein and a polyclonal antibody to the A4 core protein. For this assay, 20 PFU per cell of +L5 virions or the equivalent amount (OD_{260}) of purified –L5 virions were incubated with cells at 4°C for 1 h to permit cell attachment but not penetration. Unbound virus was removed by washing and the cells were incubated for a further 2 h at 37°C in the presence of cycloheximide. As vaccinia virus core uncoating depends on viral protein synthesis, the addition of cycloheximide results in accumulation of cytoplasmic cores and also prevents cytopathic effects resulting from viral early gene expression. Cells were fixed and permeabilized following the 4°C and 37°C incubations and incubated with antibodies. The anti-L1 antibody detected similar amounts of –L5 and +L5 virions adsorbed to the cell surface after the 4°C adsorption period, indicating that –L5 virions have no defect in cell attachment (Fig. 8). No cytoplasmic cores were detectable in –L5- or +L5-infected cells, consistent with previous observations that vaccinia virus entry does not occur at 4°C (26). Importantly, when +L5-infected cells were fixed following the 37°C incubation, numerous cytoplasmic cores were detected, whereas uncoated cores were seldom observed in –L5-infected cells (Fig. 8). Thus, although –L5 virions were able to attach to cells, they could not penetrate them.

–L5 virions are unable to induce low-pH-triggered cell-cell fusion. Vaccinia virus can induce two types of low-pH-triggered syncytium formation (10, 13). Fusion from within is triggered by briefly lowering the pH at late times of infection and is mediated by progeny enveloped virions on the cell sur-

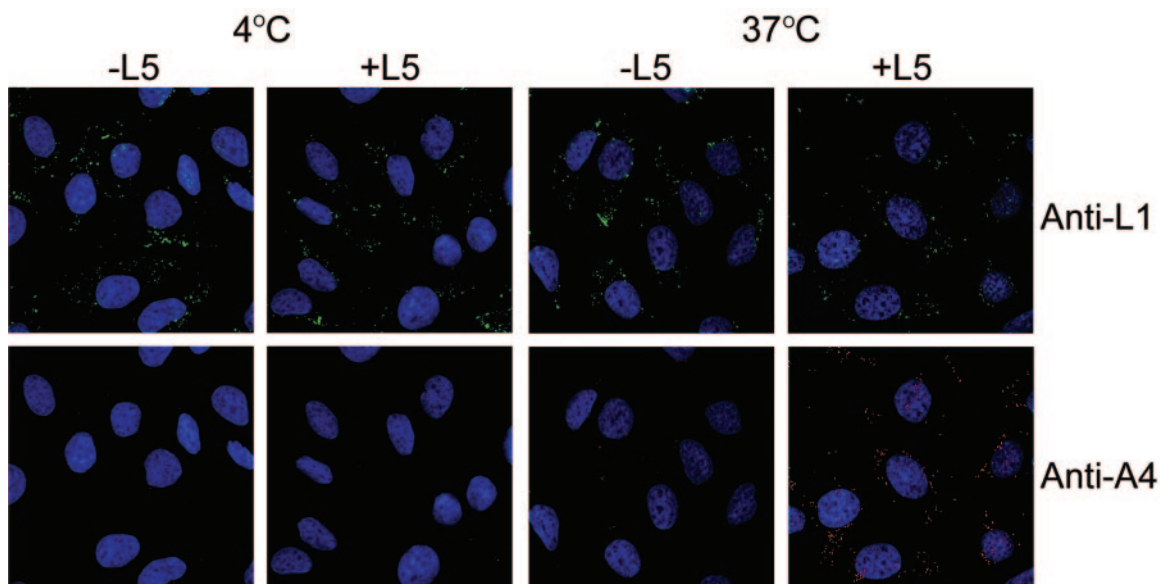


FIG. 8. Virions lacking L5 are unable to release their cores into the cytosol. Replicate HeLa cell monolayers were inoculated with 20 PFU per cell of purified +L5 virions or the corresponding OD₂₆₀ of -L5 virions at 4°C for 1 h. The cells were washed extensively and fixed or incubated for a further 2 h at 37°C in the presence of cycloheximide before fixation. Autofluorescence was quenched with 2% glycine, and cells were permeabilized with 0.1% Triton X-100. Cells were labeled with mouse anti-L1 antibody to detect intracellular mature virions on the cell surface and rabbit anti-A4 antibody to detect the A4 core protein in the cytoplasm, followed by fluorescein isothiocyanate-conjugated goat anti-mouse (green) and rhodamine red-X-conjugated goat anti-rabbit (red) antibody, respectively, as described (40). DNA was visualized by staining with DAPI. Immunolabeled cells were visualized by confocal microscopy as a sequence of optical sections, which are displayed as maximum-intensity projections.

face; fusion from without is induced by infecting cells with purified intracellular mature virions and briefly lowering the pH. In each case, syncytia form at neutral pH after the cells are incubated at 37°C. Although the mechanisms of fusion from within and without are undefined, both depend upon the same viral fusion apparatus, since virions lacking A28, H2, and A21 are defective in the ability to mediate low-pH-induced cell-cell fusion from within or without (30, 32, 40). Massive fusion from without was induced by +L5 virions, whereas no syncytia were detected in cell monolayers that had been inoculated with -L5 virions under the same conditions (Fig. 9A). Similarly, fusion from within was demonstrated in cells infected with vL5i-V5 in the presence of IPTG but not in its absence (Fig. 9B).

DISCUSSION

The discovery that A28 and H2 are required for vaccinia virus entry and fusion (30, 32) led to our identification of additional ORFs that are conserved in all poxviruses and encode proteins with related structures. The predicted protein encoded by the A21L ORF, although not homologous to A28, is similar to it in size and also has an N-terminal transmembrane domain and four conserved cysteines. The predicted product of the L5R ORF is similar to that of H2 in size and in containing a short N-terminal hydrophilic domain preceding the transmembrane domain but has only two cysteines rather than four. We recently reported that A21 is an intracellular mature virion membrane protein and indeed is required for entry and fusion (40).

Here we report that L5 is expressed late in infection and

incorporated into the intracellular mature virion membrane with the long C-terminal domain on the surface. Furthermore, the phenotype of an L5-conditional null mutant was indistinguishable from that of A28, H2, and A21 conditional null mutants. Thus, four intracellular mature virion membrane proteins are required for penetration of cells and to induce low-pH-triggered cell-cell fusion. Whether these four proteins are sufficient for entry and fusion remains to be determined. In addition to the required entry/fusion proteins, the A27, D8, and H3 proteins may participate in the initial attachment step by binding to glycosaminoglycans (5, 19, 23).

The presence of A28, H2, A21, and L5 orthologs in all poxviruses analyzed to date and the requirement for each during entry of vaccinia virus suggests that no functional redundancy exists among them. Potential roles for the proteins include receptor binding and membrane insertion. We suspect that the four proteins physically interact directly or indirectly, as we could coimmunopurify A28 and H2 (30). Nevertheless, these proteins insert into the viral membrane independently of each other, as shown here for L5 and previously for H2 and A28 (30). The requirement for four proteins to induce virus-cell membrane fusion is unusual, and only members of the *Herpesviridae* are known to have similar complexity (38). Orthologs of three glycoproteins, designated gB, gH, and gL, are thought to be essential for entry of all herpesviruses. For at least some herpesviruses, gB is a homodimer or trimer and gH and gL form a heterodimer. In addition to the three basic fusion proteins, some herpesviruses require additional nonconserved receptor-binding proteins, such as gD for most alpha-herpesviruses.

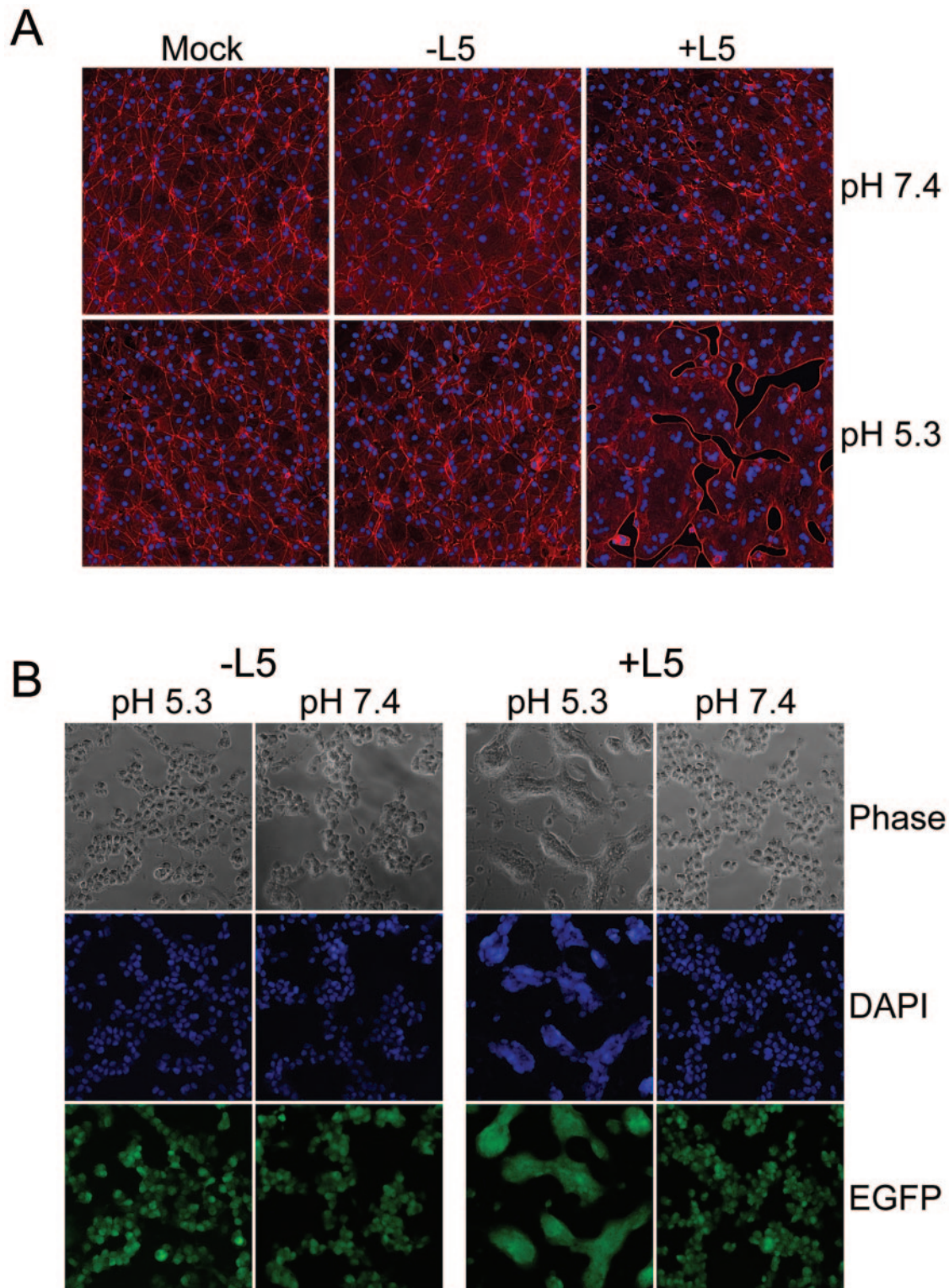


FIG. 9. Virions lacking L5 are unable to mediate low-pH-triggered cell-cell fusion. (A) Fusion from without. BS-C-1 cells were inoculated with either 200 PFU per cell of purified +L5 virions or the corresponding OD₂₆₀ of -L5 virions at 4°C for 1 h. The cells were immersed in pH 5.3 or pH 7.4 buffer at 37°C. After 3 min, the buffers were replaced with culture medium containing 300 µg of cycloheximide per ml and incubated for 3 h at 37°C. The cells were then fixed and stained with Alexa Fluor 568-phalloidin and DAPI to display actin filaments and DNA, respectively. Confocal microscopy images are shown. The extensive actin rearrangement helps to visualize the syncytia. (B) Fusion from within. BS-C-1 cell monolayers were infected with 5 PFU per cell of vV5-L5i in the presence or absence of 50 µM IPTG for 18 h and transiently treated with either pH 5.3 or pH 7.4 buffer as above before the buffer was replaced with culture medium. Cells were stained with DAPI and visualized by phase and fluorescence microscopy. vV5-L5i constitutively synthesizes EGFP in the presence or absence of IPTG, and its cytoplasmic distribution served as an indicator of cell-cell fusion.

Fusion proteins of viruses exist in a prefusion or metastable state and are usually activated by binding to a receptor on the cell surface or a pH change in endosomes (12). The situation for poxviruses is not yet clear, as no poxvirus-specific cell receptors have been identified. Ichihashi (21) reported that intracellular mature virions exist in a protected and activated form and that the infectivity of the former can be enhanced by protease treatment. There are reports that entry of released extracellular enveloped virions is dependent on a low-pH endosomal pathway, whereas intracellular mature virions entry is not (20, 41). Nevertheless, low pH can enhance intracellular mature virion entry (20) and is required for intracellular mature virion-induced fusion from without (10, 13). Moreover, our present and previous (30, 32, 40) studies demonstrate that the same fusion apparatus is required for entry of intracellular mature virions, virus spread by cell-associated extracellular enveloped virions, and low-pH-mediated fusion from within and without.

It is difficult to reconcile all of the observations in a simple model. However, it is possible that intracellular mature virions are normally receptor activated on the cell surface but that low pH can bypass this requirement. Moreover, low pH may trigger syncytium formation by accelerating and synchronizing the fusion of intracellular mature virion particles on the cell surface. In this model, syncytia form in two steps: intracellular mature virions fuse with one cell, thereby incorporating viral membrane proteins (some still in the prefusion state) into the plasma membrane, and then the membrane of that cell promotes fusion with a neighboring cell. The process is repeated, resulting in massive syncytia. Fusion from within is essentially the same as fusion from without except that the low pH also facilitates the removal of the cell-associated extracellular enveloped virion membrane to expose the intracellular mature virions.

We are hopeful that the identification of poxvirus entry proteins will facilitate further investigations on the putative cell receptor and the mechanism of fusion.

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